Cloning and Physical Mapping of Enteric Adenoviruses (Candidate Types 40 and 41)

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We have studied the DNAs of fastidious enteric adenoviruses recovered from the stools of infants with gastroenteritis. By endonuclease analysis, the strains examined represent candidate adenovirus types 40 and 41, which are thought to comprise new adenovirus subgroups F and G. Cloning of DNA from representative enteric adenovirus isolates, together with hybridization and subcleavage analysis, permitted the mapping of restriction enzyme cleavage sites. Although the restriction profiles are different for the two strains, they appear to have several cleavage sites in common. Cross hybridization studies show considerable homology between the subgroup F and G strains but much less homology to adenovirus 2. In addition, regions on both ends of enteric adenovirus genomes (map units, 2.9 to 11.3 and 75 to 100) possess little or no homology to adenovirus 2. Restriction enzyme digests reveal submolar fragments that map to the terminal regions of the genome. Electron micrographic studies of denatured and renatured DNA strands suggest that the submolar fragments may derive from cleavage of defective molecules. Inverted terminal repeat sequences were shown to comprise 0 to 3.2% of the length of complete (≥22 megadaltons) enteric adenovirus DNA molecules but 4 to 69% of incomplete-length (<22-megadalton) molecules.

Enteric adenoviruses (EAds), which are fastidious adenoviruses (Ads) found in stools, have been associated with gastroenteritis in infants (2, 9, 19, 30). Their polypeptide and restriction endonuclease patterns appear sufficiently different from those of other known human Ads, which comprise subgroups A through E, to suggest that they be classified into two new Ad subgroups, F and G (27; G. Wadell, I. Uhnoo, M. Johansson, G. Sundell, and L. Svensson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, S35, p. 240). More recently, prototype EAd F and G isolates have been tentatively designated as candidate types 40 and 41, respectively (9). Little work had been done on these agents, mainly because they do not replicate efficiently in conventional cell lines, but their growth in 293 cells (23) has facilitated investigation and has suggested a similarity to Ad host range mutants (13). In preparation for the study of the nature of this growth restriction, we have constructed physical maps of the EAd genomes and oriented them to maps of Ad2.

MATERIALS AND METHODS

Cells. Monolayer cultures of 293 cells were grown in Eagle minimal essential media with Earle balanced salts supplemented with 10% heated fetal calf serum, glutamine, aureotetracycline, penicillin, and streptomycin.

Viruses and viral DNAs. EAd F and G strains were isolated from stool specimens of children with diarrhea at Children's Hospital National Medical Center, Washington, D.C., and identified by neutralization and restriction endonuclease analysis. A prototype G strain (1105) which was used for cloning was provided by C. Brandt and A. Z. Kapikian and passed at least 20 times in 293 cells, as previously reported (23). The cloned EAd F strain was a gift of J. C. de Jong (VR 931 in the American Type Culture Collection, Rockville, Md.). Ad2 was obtained from H. Raskas and grown in KB spinner culture as previously described (21). EAd and Ad2 virions were purified, and DNA was isolated by protocols described previously for Ad2 (26).

Cloning. pBR325 and pBR322 were gifts of G. Vande Woude. DNA ligase was purchased from Bethesda Research Laboratories, Gaithersburg, Md. DNA extracted from EAd G 1105 was cut with EcoRI or BamHI and ligated into pBR325 digested with the same enzyme. In addition, EAd F or G DNAs were digested with Smal and ligated into pBR322 at the PvuII site. Recombinants were transfected into Escherichia coli LE 392 as previously described (15). Plasmids were grown, and their DNA were extracted by a modification of the procedure of Birnboim and Doly (1). Cloned EAd G fragments studied include EcoRI-B,-C, and -D; BamHI-A and -B; and Smal-B1, -B2, -C, -D, -E, and -F. Cloned EAd F fragments used in this study include Smal-D, -E1, and -F2.

Endonuclease Analysis. Restriction endonucleases were purchased from Bethesda Research Laboratories and used as recommended by the supplier. Double-enzyme digestions were performed by cleaving DNA with both enzymes simultaneously. Phage lambda DNA for use as sizing markers were obtained from Bethesda Research Laboratories. Varicella zoster virus DNA, prepared as previously described and cut with EcoRI, was also used for sizing (22). Digests were analyzed by agarose gel electrophoresis in horizontal slab gels of 0.9% agarose submerged in E buffer (40 mM Tris buffer, 20 mM sodium acetate, 1 mM EDTA, pH 7.4) containing 0.5 μg of ethidium bromide per ml. DNA bands were visualized with a UV lamp and photographed with a Polaroid camera and type 52 film.

DNA hybridization. Nick translation was performed with Bethesda Research Laboratories kits and [32P]dCTP (specific activity of 400 or 3,000 Ci/mmol) obtained from Amersham Corp., Arlington Heights, Ill. Southern blotting was used to transfer DNA from agarose gels to nitrocellulose, and hybridizations were performed with 50% formamide at 42°C as described previously (28). Some hybridizations used EAd or Ad2 DNA restriction fragments, which were recovered either from preparative gels by the “freeze squeeze” technique (24) or from low-melt agarose as described previously (29).

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Electron microscopy. Procedures for electron microscopy and denaturation-reannealing studies have been previously described (10).

RESULTS

The restriction endonuclease digestions shown in Fig. 1 revealed that, although they had similar patterns with several enzymes, representatives of subgroups F and G possess unique cleavage profiles. The estimated sizes of the fragments cut with these enzymes and the total genomic weights for the digestes were derived by comparison with known sizes of cleavage fragments of Ad2, phage lambda, and varicella zoster virus DNAs (Table 1).

The strategy for mapping the EAd F and G DNAs involved several steps. Individual restriction fragments were cloned in plasmid vectors and used as hybridization probes to identify homologous fragments in virion DNAs cleaved with different enzymes, as in the upper right portion of Fig. 1. Digestion of the plasmid-EAd DNA recombinants with enzymes other than those used in cloning or with two enzymes simultaneously permitted localization of the cleavage sites for the other enzymes within the cloned fragments.

Orienting the EAd DNA maps with respect to the Ad genomes was accomplished by hybridizations to purified Ad2 DNA fragments. The results of all the above studies permitted construction of the cleavage maps shown in Fig. 2.

Elucidation of the physical maps permitted examination of the homology between the EAd F and G genomes and comparison with Ad2 DNA. A series of cross-hybridization studies, one of which is shown in Fig. 3, were used to construct crude composite homology maps (Fig. 4). Although the degree of cross homology could be more precisely quantitated by reassocation kinetic analyses at a range of stringencies, our data nonetheless reveal substantial homology between the F and G strains throughout most of the length of the genome but considerably less homology to whole Ad2 DNA. In addition, there are regions at both the right and left ends of the EAd genomes which appear to have almost no homology to Ad2, and the same regions in the Ad2 genome seem to have little or no homology to the EAd genome. These regions include the Ad2 Smal fragment E on the left end and fragments C, G, and K on the right end.

Construction of restriction maps was complicated by a lack of uniformity of some cleavage fragments. Some bands

FIG. 1. Agarose gel electrophoresis and Southern hybridization of EAd F and G DNAs cleaved with six different restriction endonucleases. EAd F digestion fragment pairs with asterisks (e.g., C and C*) appear to be submolar bands representing alternate forms of fragments mapping to the left end of the EAd F genome (Fig. 2 and 5). Some lanes containing EAd F or G digests demonstrate smeared fragments (e.g., Smal A, Kpnl A, Xhol B), which seem to be comprised of a family of discrete bands and map to the right end of the genome (Fig. 2). The upper right panel is an autoradiogram of the Southern-transferred agarose gel on the left after hybridization to in vitro 32P-labeled pBR325 containing the EAd G EcoRI fragment B.
TABLE 1. Estimated sizes in base pairs of restriction fragments of EAd strains from subgroups F and G

<table>
<thead>
<tr>
<th>Fragment</th>
<th>BamHI</th>
<th>EcoRI</th>
<th>HindIII</th>
<th>KpnI</th>
<th>SmaI</th>
<th>XhoI</th>
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<tr>
<td></td>
<td>F</td>
<td>G</td>
<td>F</td>
<td>G</td>
<td>F</td>
<td>G</td>
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<td>12,900</td>
<td>16,000</td>
<td>16,000</td>
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<td>9,500</td>
<td>6,100</td>
<td>6,000</td>
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<td>7,000</td>
<td>6,100</td>
<td>4,350</td>
<td>5,200</td>
<td>4,300</td>
</tr>
<tr>
<td>D</td>
<td>4,100</td>
<td>1,320 (D₁); 1,320 (D₂)</td>
<td>2,600</td>
<td>3,150</td>
<td>4,300</td>
<td>3,150</td>
</tr>
<tr>
<td>E</td>
<td>2,050</td>
<td>850</td>
<td>2,650</td>
<td>2,600 (E₁); 2,600 (E₂)</td>
<td>2,600</td>
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<td>2,400</td>
<td>2,550</td>
<td>1,810</td>
<td>1,800</td>
</tr>
<tr>
<td>G</td>
<td>500</td>
<td></td>
<td>2,130</td>
<td>1,660</td>
<td>1,320</td>
<td>550</td>
</tr>
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<td>830</td>
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<tr>
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<td>850 (I₁); 850 (I₂)</td>
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<td>1,320</td>
<td>550</td>
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* These alternate fragments are believed to derive from the complete, nondefective molecules and, therefore, are used in calculations of the estimated genome size.

FIG. 2. Physical maps of fragments generated by cleavage of EAd F and G DNAs with restriction enzymes BamHI, EcoRI, HindIII, KpnI, SmaI, and XhoI.
FIG. 3. Southern hybridizations demonstrating cross homology between EAd F and EAd G and Ad2 DNAs. The ethidium bromide-stained agarose gel on the right contains Smal digestions of viral DNAs from EAd G, EAd F, and Ad2 (adeno subgroup C). Replicates of this gel were Southern transferred and hybridized to in vitro 32P-labeled Ad2, EAd G, or EAd F DNAs and autoradiographed for 4 days. The letters below the figure denote the probes.

(Fig. 1), EAd G Smal-A, KpnI-A, or BamHI-B, were consistently faint or smeared relative to other bands and were accompanied by a family of slightly larger fragments just above them. Some other bands, although distinct, also appeared to have less than a molar intensity (Fig. 1), i.e., EAd F HindIII-C and -C* or KpnI-D and -D*. Still others were not consistently present or were present in low concentration and were visualized only by hybridization to homologous cloned fragments. Hybridization studies and the completed restriction maps eventually showed that the smeared bands, or bands with a family of fragments, are from the genome termini, and each component of the family of bands appeared to differ by distinct DNA additions or deletions.

One possible explanation for the submolar fragments relates to the method of virus growth and purification used in our studies. Because relatively small amounts of virus could be obtained from infected 293 cell cultures, we attempted in many cases to maximize the yield of DNA by performing isopycnic centrifugation in CsCl only once and harvesting the lighter-density bands (1.30 g/ml) along with the main virus band (1.34 g/ml). We previously reported that virus isolation by CsCl gradients yielded at least two bands of EAd particles, the lighter of which was shown by electron micro-

copy to contain empty virions, and particles with DNA of less than whole genomic length (23). Further electron microscopic studies of DNA from these incomplete particles revealed wide heterogeneity in the size and organization of the DNA molecules. Upon denaturation and reannealing, single strands of viral DNA formed duplex panhandle structures, presumably by intramolecular annealing of inverted terminal repetitions. Full-length molecules (≥22 megadaltons) possessed short panhandles, comprising 0 to 3.2% of the genome, whereas the shorter (<22 megadaltons) molecules had long panhandles, constituting as much as 69% of the DNA molecule (Fig. 5 and 6). Thus, our purification procedures permitted inclusion of defective genome frag-

FIG. 4. Composite mapping of relative hybridization of EAd G to EAd F (above) and to Ad2 (below). Maps were generated by rating on an arbitrary 0 to 4 scale the relative degree of intensity of the bands in overnight autoradiographs or on a 0-to-1 scale for 4- to 7-day autoradiographs in experiments such as those shown in Fig. 3.

FIG. 5. Electron micrograph showing denatured and reannealed DNA molecules extracted from EAd G virions banding in CsCl at a density of 1.299 g/ml. Arrows denote the presumed internal end of the duplex structures.
ments. The intensities of the submolar bands were lower in gels containing DNA recovered from the denser band alone (data not shown).

A second explanation for the submolar bands could involve the method of viral passage employed. To build up adequate stocks of virus, the EAds were passaged repeatedly at relatively high multiplicity. DNA from lower-passage material seemed to exhibit fewer and less intense redundant bands (data not shown).

The lengths of defective genome fragments were not randomly distributed, however. There were distinct subpopulations of defective molecules. This is seen quite clearly in studies of DNA isolated from only the most dense band of the seventh passage of an EAd F isolate, "Dab" (Fig. 7). Hybridizations in which the left terminal EAd G SmaI-B, fragment was used as a probe revealed two homologous submolar fragments in each of the BamHI, HindIII, and KpnI digests of the EAd F isolate, labeled D, D*; C, C*; and D, D*, respectively. The lower of these two bands in the HindIII lane appears brighter in the ethidium bromide-stained gel because it overlaps another fragment in the same location.

**DISCUSSION**

Although endonuclease analysis and mapping show clear differences between the EAd F and G strains, there are several properties that suggest that they are fundamentally very alike: the degree of homology between them, their common ability to grow in 293 cells far more efficiently than in conventional cell lines, the presence within each strain of specific genomic regions lacking homology to the Ad2 genome, and their association with gastroenteritis (2, 9, 19, 30; Wadell, G., et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1982).

The cross-hybridization studies are interesting because of the particular functions which map in Ad2 genome areas lacking homology. The region on the right end (map units, ca. 75 to 100) includes the area which in Ad2 codes for fiber protein, which is required for cell attachment (5). These differences may reflect the apparent tropism of EAds for intestinal cells. The sequences at the left which lack homology to Ad2 DNA (map units, ca. 2.9 to 11.3) include at least part of the early region 1 coding sequences. Differences in this region of the genome may relate to the early replicative block of EAds in conventional cell lines and the possible complementation of defective early EAd genes by the Ad5 genes expressed in 293 cells (23).

Incomplete adenovirus particles have been noted for several years, and their nature, origin, and significance have been discussed in relation to theories of Ad DNA replication (4, 7, 12, 16, 17, 20, 25). Our observations are similar to those reported in studies of incomplete particles of other Ad types. In those studies, analysis of intracellular viral DNA has revealed incomplete DNA molecules which extend various distances from either end. However, the selective packaging of molecules extending from the left end, but lacking variable length sequences from the right end, also seen with EAds, is thought to be related to a packaging function mapping in the left end of the genome (8, 11). Similar to the EAds in our present observations, discrete subgenomic species of conventional Ads (6) have been noted, and long panhandle structures formed by intramolecular annealing have been observed in denaturation-renaturation electron microscopic studies of incomplete particles (3, 14, 18).

Although there has been speculation on the origin of incomplete particles, any postulation of a random premature termination of replication would fail to account for the
families of multiple distinct terminal fragments that we have seen (Fig. 1 and 7), nor could it explain the formation of the long-reiterated sequences capable of forming panhandle structures. Similarly, theories concerning a fold back during DNA replication could explain the long panhandles and truncated molecules, but they cannot explain the nonrandom generation of multiple species of terminal fragments. Such observations suggest an alternative or additional process, resulting in reiterations or segmental deletions and perhaps involving preferential sites of DNA polymerase dissociation or direction change at replication forks.

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LITERATURE CITED


